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Allele-Specific Conditional Destabilization of Glutamine Repeat mRNAs

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Several late-onset neurological diseases are caused by the inheritance of an expanded CAG repeat coding for polyglutamine. To date there is no effective means of halting the progression of these diseases, and their underlying molecular mechanisms remain a mystery. Strategies designed to specifically reduce the levels of long repeat mRNA might provide an effective therapy for these diseases. An emphasis on allele specificity is necessary to avoid the potential toxicities associated with reduction of expression. The experiments described here are based on the relationship between translation and mRNA stability and the idea that translation of a repeated codon might be extremely sensitive to reductions in levels of cognate aminoacylated tRNA. Consistent with this hypothesis, we have discovered that reduced glutamine concentration destabilizes mRNAs coding for long glutamine repeats while sparing short repeat versions of the same mRNAs. These results suggest therapy might be attained with existing compounds or environmental conditions known to decrease free glutamine levels.

Key words: Polyglutamine; Huntington's disease; Glutamine; tRNA; Translation; mRNA stability; Rational therapeutic

SEVERAL neurological disorders are caused by the inheritance of a long CAG repeat coding for polyglutamine. Huntington's Disease (HD), the most prevalent of these disorders, is inherited as an autosomal dominant trait. Symptoms are neurological and psychiatric, involving involuntary movements and cognitive impairment (36). Symptoms typically present during mid life (early 40s) and progress until death 15–20 years after onset. The expanded disease allele is between 36 and 180 units in length within exon 1 of a gene of unknown function called huntingtin (20,39). Longer repeats are associated with earlier ages of onset and short repeats (less than 36 CAGs) are found in individuals not affected by HD (22). The repeat codes for a polyglutamine stretch near the N-terminus of the huntingtin protein, which contributes to protein aggregates found in affected regions of patients' brains (14,30). The other inherited late-onset neurological disorders caused by long CAG/polyglutamine repeats include spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and several of the spinocerebellar ataxias (SCAs) [reviewed in (12)]. The molecular mechanisms underlying HD and the other CAG/polyglutamine disorders remain unknown.

For each of these diseases the molecular mechanism of pathogenesis may involve multiple pathways. For HD this view is supported by the existence of many interacting partners of the huntingtin protein and the potential to partner with many other proteins by polyglutamine–polyglutamine interactions (6,18,21, 23,26,45). Many processes have been reported to be affected by HD including mitochondrial function (34), transcription (9), glutamate uptake by vesicles in neurons (25), and vesicular transport (19). This diversity might make HD difficult to treat by rational drug design specifically targeting each of these processes. The problem of molecular diversity underscores the advantage of potential therapies designed to reduce *HD* gene expression, because such strategies might reverse the effects of the mutation on all affected processes. For HD this approach is further supported by evidence that the mutation causes pathology by a gain-of-function mechanism (1).

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The benefits of reducing huntingtin expression have been shown in animal models by the comparison of transgenic mice where higher levels of expression correlate with an earlier onset of HD-like symptoms (29,37). Furthermore, eliminating expression by the use of a conditional transgene reverses an HD-like pathology in mice (48). It is not yet clear the degree of reduction of expression that would be needed to provide therapeutic benefit. Nevertheless, it is possible that slight reductions in expression over the decades that typically precede symptoms may be helpful or entirely preventative.

The major drawback of downregulation strategies in general is the possibility that a gene product is essential. For example, *Hdh* (the mouse homolog of the *HD* gene) is necessary in development and knockout mice exhibit lethality early in embryogenesis (16, 27,50). Reducing huntingtin protein expression to less than 30% of wild-type levels impairs neurogenesis (46), and removal of the *Hdh* gene from the forebrain late in development (5 days postpartum) causes progressive neurodegeneration (13). Chimera analysis showed murine embryonic stem (ES) cells lacking *Hdh* gene expression did not contribute to some brain regions. Furthermore, these chimeras showed a number of abnormalities (38). These results suggest that a downregulation strategy, if carried too far, might cause harm to some brain regions. Nevertheless, loss of only one *Hdh* allele, which is known to decrease brain expression to approximately half of normal, is compatible with normal development and neurological function in mice (16,50). Furthermore, humans with balanced translocations through the *HD* gene lack abnormalities (1). Because almost all HD patients have one mutant and one normal copy of the HD gene, an opportunity exists for decreasing the expression of the mutant allele while preserving expression of the wild-type allele.

This goal might be achieved by one of several sequence-based reduction-of-expression methods. These include antisense RNA, ribozyme, DNA enzyme, and RNA interference methods (5,7,35,49). RNA interference (RNAi) is currently the most popular of these methods and several advancements in our understanding of the machinery involved in RNAi have brought this method closer to providing a therapy (41). The defining difference between the disease and wild-type alleles is the repeat length. Thus, the repeat would be an ideal sequence target for discrimination between alleles. Thus far RNAi has not been effective directly against long CAG repeats (7,32). This might be due to an unusual structure of the repeats in mRNA or simply that CAG is not a sequence that is recognized by the RNAi machinery of mammalian cells. Miller et al. have recently reported an allele-specific RNAi therapy that targets differences other than the CAG repeat. This indirect targeting results in reductions of CAG repeat containing RNAs in mammalian cells (32). Because most sequences are not amenable to RNAi, it is not clear how many transcribed polymorphisms will be useful for allele-specific reduction of expression. Such sequences must be present as heterozygosities in many patients to be generally useful. The successful application of this approach will require customization for each patient. Take, for example, HD, where the expanded CAG repeat is present on multiple haplotypes (31). Thus, targeting a heterozygosity would require the determination of which transcript variant contains the expanded allele for each patient—not a simple task given the great length of the HD mRNA. The other major obstacle for effective therapy is delivery of oligonucleotide. These challenges suggest this indirect strategy will require many more years of study and then apply only to those lucky enough to have the right combination of linked variants.

We reasoned that discrimination of mRNAs with different repeat lengths might be achieved by exploiting differences that would occur during translation of the CAG/polyglutamine tracts. Supplying the ribosome with enough charged glutamine-tRNA should be more challenging with increasing repeat length. As in the case when rare codons are present in an mRNA, limiting charged tRNA is known to induce ribosome pausing, which can result in mRNA destabilization (24). We provided conditions designed to reduce the cell's ability to replenish charged glutamine-tRNA (10) while monitoring the levels and stability of mRNAs with different CAG/polyglutamine repeat length.

We present results showing that reducing glutamine concentration destabilizes mRNAs coding for a long glutamine repeat while sparing short repeat versions of the same mRNAs. We monitored an mRNA-rich in glutamine codons that lacked a long repeat. This mRNA's stability was not affected by glutamine deprivation, suggesting that this strategy will spare other essential cellular transcripts rich in distributed glutamine codons. Thus, therapy might be attained with existing compounds or environmental conditions known to decrease free glutamine levels (42). This work provides the foundation for the rational design of more specific therapeutics that target the translation machinery.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Gene targeted ES cell lines expressing repeats of 150 glutamines were described previously as $Hprt^{Q150}$

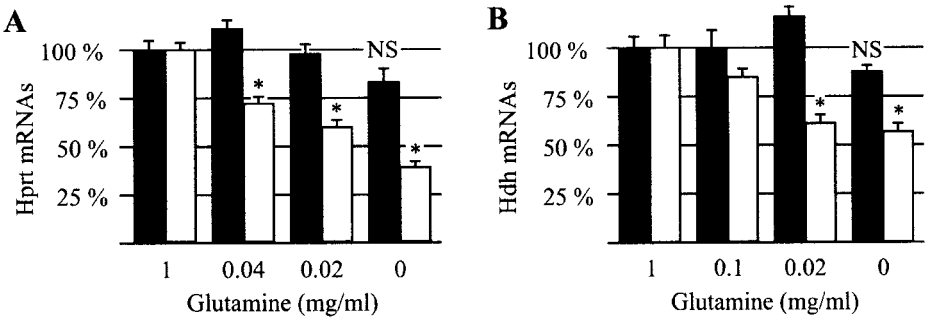

Figure 1. Glutamine deprivation reduces long repeat mRNA levels. Steady-state levels of Hprt (A) and Hdh mRNAs (B) as determined by quantitative real-time PCR. Filled bars represent wild-type mRNA levels and open bars represent mRNA levels of Q150 knock-in alleles. Error bars represent SEM of 6–9 replicates. Asterisks indicate significant differences in comparison to wild-type at the same glutamine concentration and a significant difference from the Q150 allele mRNA level for 1 mg/ml glutamine ($p < 0.004$, Mann-Whitney). "NS" indicates no statistical difference compared to wild-type mRNA level from cells in 1 mg/ml glutamine.

(33) and Hdh^{Q150} (28) lines. *Hprt* alleles are hemizygous, because they are located on the X-chromosome in male ES cells. However, construction of Hdh hemizygotes required an additional gene targeting reaction to remove the promoter and exon 1 of the wild-type copy from heterozygous long repeat ES cells, performed as described previously (8). We have confirmed that these cells express only the long repeat version of Hdh and lack wild-type Hdh mRNA by RT-PCR across the repeat region. Lines hemizygous for the wild-type *Hdh* allele described in Cearley and Detloff (8) were used as controls. ES cells were expanded in standard ES cell media supplemented with leukemia inhibiting factor as described (8) except murine embryonic fibroblasts were not included in the last passage. Medium lacking glutamine was prepared by replacing DMEM with DMEM lacking glutamine and by replacing serum with dialyzed fetal calf serum. The glutamine synthetase inhibitor methionine sulfoximine (MSO, Sigma) was supplemented at 10 mM and glutamine was added to the concentration specified. Rate of mRNA decay was determined by growing the cells in medium containing the appropriate levels of glutamine for 2 h followed by the addition of a transcription inhibitor. Actinomycin-D (Sigma) at 10 μM (shown here) and α-amanitin poly-lysine (Sigma) at 0.4 μM (data not shown) provided similar results.

RNA Preparation and Quantitative Real-Time PCR

RNA was prepared using Trizol reagent (Invitrogen); 0.1 μg was reverse transcribed in a 20-μl reaction using Applied Biosystems's High Capacity cDNA Archiving Kit following the manufacturer's protocol. The cDNA was diluted 1:150 for analysis by TaqMan real-time PCR. The Hprt target mRNA was measured with the primers AGGAC CTCTC GAAGT GTTGG ATAC and GGGCA TATCC AACAA CAAAC TTG and the TaqMan probe

TABLE 1
POSITION AND NUMBER OF GLUTAMINES FROM DIFFERENT ALLELE PRODUCTS

Allele	Positions of Gluatmines in Protein	Total Codons	Q Codons			Longest Q Repeat
			CAG	CAA	Total	
Hdh		3120	138	35	173	7
Hdh^{Q150}		3263	282	34	316	150
Hprt		219	2	1	3	1
$Hprt^{Q150}$		380	152	1	153	150

Protein represented by box with N-terminus on left. Vertical lines represent glutamine residues with relative positions drawn to scale. Longer lines represent positions of glutamines from the more rarely used CAA codon.

AGGCC AGACT TTGTT GG. The Hdh target mRNA was measured with the primers GACCG TGTGA ATCAT TGTCT AACAA and GATGC CCAAG AGTTT CTGAA ATTC and the TaqMan probe CAGTC TCTCA GAAAT T. Applied Biosystems 18s rRNA and β-actin mRNA assays were used as controls. Target probes were labeled with FAM and control probes were labeled with VIC, allowing multiplex reactions. Data were calculated using the ΔΔCt method after validation as described by the manufacturer.

RESULTS

Knock-in mouse ES cells hemizygous for alleles expressing repeats of 150 glutamines from either *Hprt* or *Hdh* were given varying levels of glutamine while inhibiting cellular glutamine synthesis. Reduction of glutamine resulted in graded decreases in steady-state levels of long repeat mRNAs without significantly reducing wild-type mRNA levels (Fig. 1). Thus, high glutamine levels, which are not normally required for the maintenance of mRNAs, are required when those mRNAs code for long glutamine repeats.

Sensitivity to glutamine deprivation did not correlate with the total number of glutamine codons in these mRNAs. Comparison of the effective reduction of $Hprt^{Q150}$ mRNA, which has 150 of its 153 glutamine codons in a repeat, with the lack of an effect on wild-type Hdh mRNA, which has 173 dispersed glutamine codons (Table 1), indicates the effect depends on the presence of a long repeat rather than total glutamine codon content ($p = 0.0004$, Mann-Whitney). These results are analogous to decreases in an mRNA's translatability when rare codons are clustered rather than dispersed (43). Thus, strategies based on the glutamine deprivation effect might avoid diminishing the levels of other essential mRNAs rich in distributed glutamine codons.

Comprehending the mechanism underlying the

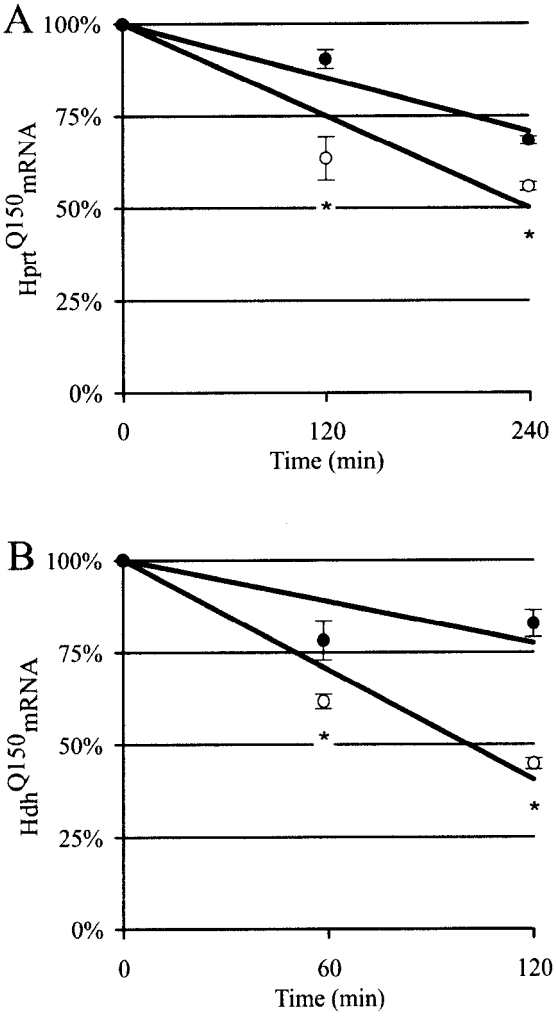

Figure 2. Glutamine deprivation destabilizes long repeat mRNAs. Levels of $Hprt^{Q150}$ (A) and Hdh^{Q150} (B) mRNAs remaining after inhibition of transcription with actinomycin-D (at time zero) in 1 mg/ml glutamine (filled circles) or 0 mg/ml glutamine medium (open circles). Error bars represent the SEM of nine measurements. Asterisks indicate significant difference between long repeat mRNA levels at 1 and 0 mg/ml glutamine ($p < 0.0001$, Mann-Whitney).

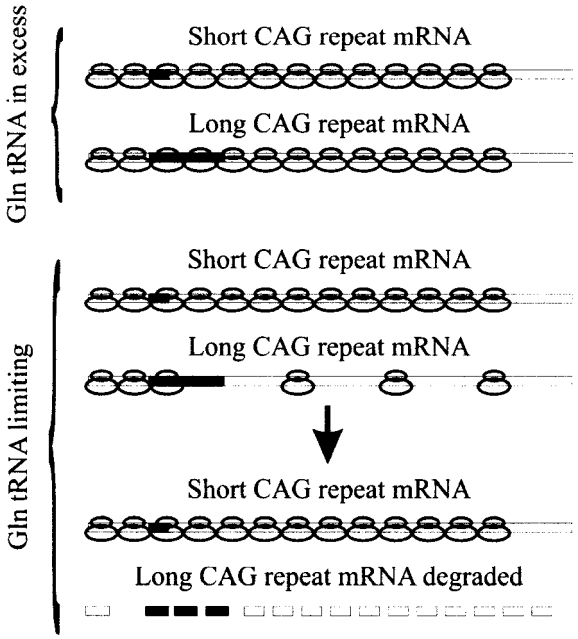

Figure 3. The pausing induced decay model (PID). A proposed mechanism to explain reduction of long repeat mRNA levels by glutamine deprivation. Open boxes represent mRNAs and black boxes represent CAG repeat regions coding for glutamine. Each double oval represents a translating ribosome. Under normal conditions where charged glutamine-tRNA is abundant (upper panel) translation elongation is not rate limiting. Removal of glutamine could cause a decrease in charged glutamine-tRNA levels, resulting in ribosome pausing during translation of long repeats, but not during the translation of short repeats. The paused ribosome allows mRNA degradation of long repeat mRNAs.

glutamine deprivation effect will provide additional targets for refinement and improvement of therapeutic strategies. We therefore tested the stability of short and long repeat mRNAs under conditions of glutamine deprivation. Figure 2 shows the relative levels of Hdh^{Q150} and $Hprt^{Q150}$ mRNAs after inhibition of transcription. Glutamine deprivation resulted in reduced mRNA stability for both long repeat mRNAs. In contrast, wild-type versions of these mRNAs without long CAG repeats were unaffected by glutamine withdrawal (data not shown), a result consistent with the lack of effect on their steady-state levels (filled bars in Fig. 1). Thus, our ability to induce an allele-specific decrease in the steady-state levels of long repeat mRNAs is, at least in part, due to enhanced mRNA decay.

DISCUSSION

We have found conditions that destabilize mRNAs coding for long glutamine repeats. Our current model for how this process occurs, shown in Figure 3, relies on ribosome pause induced decay (PID). This model and the data presented here are consistent with mechanisms of mRNA degradation known to involve ribosome pausing. Precedent for such a model is drawn from the several well-studied mRNA decay pathways involving a paused or stalled ribosome. For example, translational pausing is an important means of regulating the stability of c-*myc* mRNA. This mRNA has an endonuclease cleavage site called the CRD (coding region determinant). Rare codons upstream of this region that cause ribosomal pausing, presumably due to limiting cognate tRNA levels, are essential to the destabilizing effects of the c-*myc* CRD. One means of limiting the amount of a particular charged tRNA is to deprive the cell of the amino acid needed in the aminoacylation reaction (10). Thus, glutamine deprivation might cause repeated glutamine codons to also act as ribosome pause sites. Hdh mRNA has a short region of significant homology the c-*myc* CRD region (Fig. 4). Hprt mRNA has no such homology, but may have other as yet uncharacterized destabilizing sequences.

The glutamine codon CAG is an abundant one in transcripts from mice and humans (2), suggesting that the cell has an abundant supply of charged glutamine tRNA-CAG. Under normal conditions charged glutamine-tRNA is abundant enough to support efficient translation of long repeat versions of the HD protein, because its levels are approximately equivalent to those of wild-type huntingtin protein in both HD patients (40) and knock-in mice (28). Nevertheless, the long repeat causes a mild (30%) reduction in Hdh^{Q150} mRNA when compared to wild-type Hdh mRNA levels of mouse brain (15). In this report we show glutamine deprivation selectively decreases long repeat mRNA levels, perhaps due to the increased requirement for charged glutamine-tRNA during the translation of long versus short repeats. The translation of a long repeat requires the continuous repetitive consumption of charged glutamine-tRNA. If this rate of consumption overtakes the rate of replenishment, the amount of charged glutamine-tRNA available for continuing the translation of the repeat will become transiently limiting, leading to slowing of elongation or a pause. In this scenario, each additional repeated codon increases the possibility of a pause within the repeat and thus the probability of destruction of the mRNA being translated.

A mathematical model incorporating these features is presented in Figure 5. This model explains how mRNAs coding for expanded glutamine repeats could be greatly affected by very small reductions in charged glutamine-tRNA levels. This model takes into account the likely scenario where there is spare capacity (or a buffer) in the levels of charged glutamine-tRNA allowing the ribosome to efficiently translate short glutamine repeats. Thus, a ribosome would be unlikely to pause until any such buffer was consumed during the translation of a long repeat. Lowering glutamine concentration would result in a diminished buffer and a lesser rate of replenishment. These factors would result in an increased probability

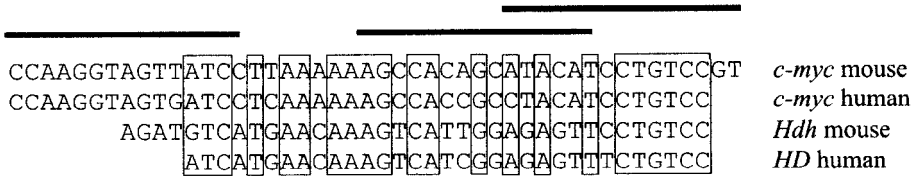

Figure 4. Alignment of homology between c-*myc* CRD region and HD coding sequence. The displayed sequence starts at nucleotide 1172 of the mouse c-*myc* mRNA, 1741 of the human c-*myc* mRNA, 8907 of the mouse *Hdh* mRNA, and 8983 of the human *HD* mRNA. Lines above the sequence indicate positions where antisense RNAs were effective at reducing the binding of the CRD binding protein to the human c-*myc* CRD region (11). Boxes represent regions of identity between human c-*myc* and human *HD* mRNAs.

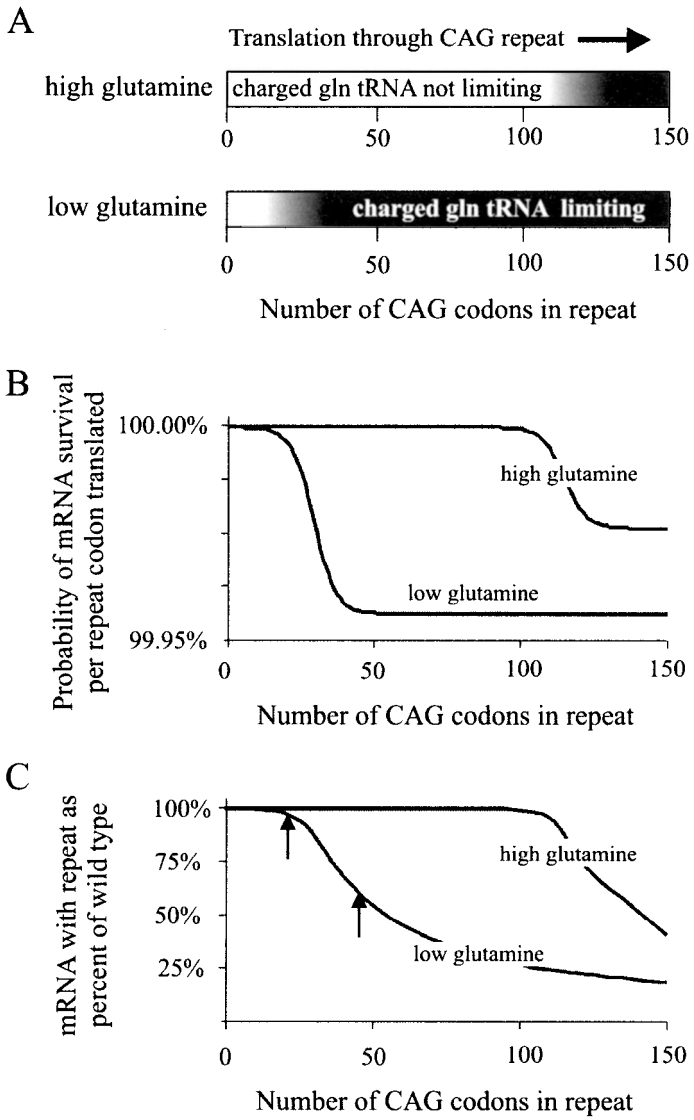

Figure 5. A mathematical model to explain the glutamine deprivation effect. Under normal conditions the amount of charged tRNAs available to a translating ribosome exceeds the rate of consumption and initiation is rate limiting for translation. The translation of a long CAG repeat starts with high levels of charged glutamine-tRNA, which is rapidly depleted during translation of the repeat to a point where its local concentration becomes transiently limiting. The resulting translational pauses would occur with greater likelihood in the downstream regions of a long repeat (represented by a darkening of the upper box in A). Limiting glutamine would have two effects. First, it would reduce the stores of charged tRNAs available at the start of translation, making the transient depletion occur after the translation of only a few repeated codons (represented by the lengthened dark area of the lower box in A). Second, the duration of each pause would be on average greater, because the ribosome would need to wait for the now scarce charged glutamine-tRNA. The probability of mRNA degradation increases with a greater probability and duration of each pause. These features have been mathematically represented in (B) by sigmoid equations of the type $Y = [1 - 1/(1 + e^{-x})]$. A left-shift due to deprivation of glutamine is indicated to take into account the enhanced probability of a pause earlier in the repeat. A lower plateau indicates where consumption due to translation (decreased by pausing) equals production by glutaminyl tRNA synthetase activity (also reduced by depriving it of its substrate, glutamine). Several functions other than the sigmoid curve would also be suitable to describe a hypothetical reduction of mRNA stability caused by pausing due to limiting charged glutamine-tRNA. Steady-state mRNA levels are typically represented by the kinetic equation $K_{TS} = K_D * X$, where K_{TS} is the rate of transcription, K_D the normal rate of mRNA decay, and X the concentration of the RNA at steady state. We add to this equation a rate of destruction due to pausing during translation (K_{TD}) to yield the equation $K_{TS} = K_D * X + K_{TD} * X$. Solving for X gives $X = K_{TS}/(K_D + K_{TD})$. For a gene with no clustered glutamine codons $K_{TD} = 0$ and its steady-state concentration $X_0 = K_{TS}/K_D$. Thus, the ratio of mRNA concentrations for a gene with repeats (X_R) to the concentration without repeats (X_0) is $X_R/X_0 = K_D/(K_D + K_{TD})$. $K_{TD} = K_i * P_D$, where K_i = the rate of translation initiation and P_D = the probability that degradation occurs due to pausing during a single translation of the repeat. $P_D = 1 - \Pi P_S$ where ΠP_S is the product of the probabilities of mRNA survival at each codon of the repeat (shown in B). Thus, the final equation is $X_R/X_0 = 1/[1 + (K_i/K_D) * (1 - \Pi P_S)]$. Notice that K_i/K_D = the average number of times a message is translated. The curves in (C) were derived from the Ps from (B), with the assumption that the number of translations per message is 180 in high glutamine and one half that rate in low glutamine. We reasoned that pausing might cause a ribosomal traffic jam, lowering the translation initiation rate.

of ribosome pausing further into the repeat. This model predicts that even a small decrease in mRNA survival per glutamine translated (to illustrate we chose a decrease of 0.05%) when compounded can have profound effects on the mRNA level when a long glutamine repeat is translated. The model predicts a transient decrease in the levels of charged glutamine-tRNA that would only occur during translation of a long repeat. Thus, long repeats would be subject to the effects of glutamine deprivation, whereas translation of distributed glutamine codons would allow time between each glutamine codon for the replenishment of charged glutamine-tRNA. This model can explain our data showing the glutamine deprivation effect depends on the presence of a CAG/glutamine repeat rather than the total glutamine codon content of an mRNA. This model also highlights the possibility that allele-specific destruction of repeat lengths typical for HD patients might be achieved. For example, given the parameters chosen for the curves in Figure 5, HD mRNAs with the median normal repeat length of 20 would be maintained at 99% of wild-type levels, whereas mRNAs with the median disease length of 44 would be reduced to 60% (arrows in Fig. 5C). Although the shapes of these curves need to be determined by empirical methods, the model highlights the potential therapeutic value of the described strategy.

The precise molecular mechanism of the glutamine deprivation effect is not yet known. Potential mecha-

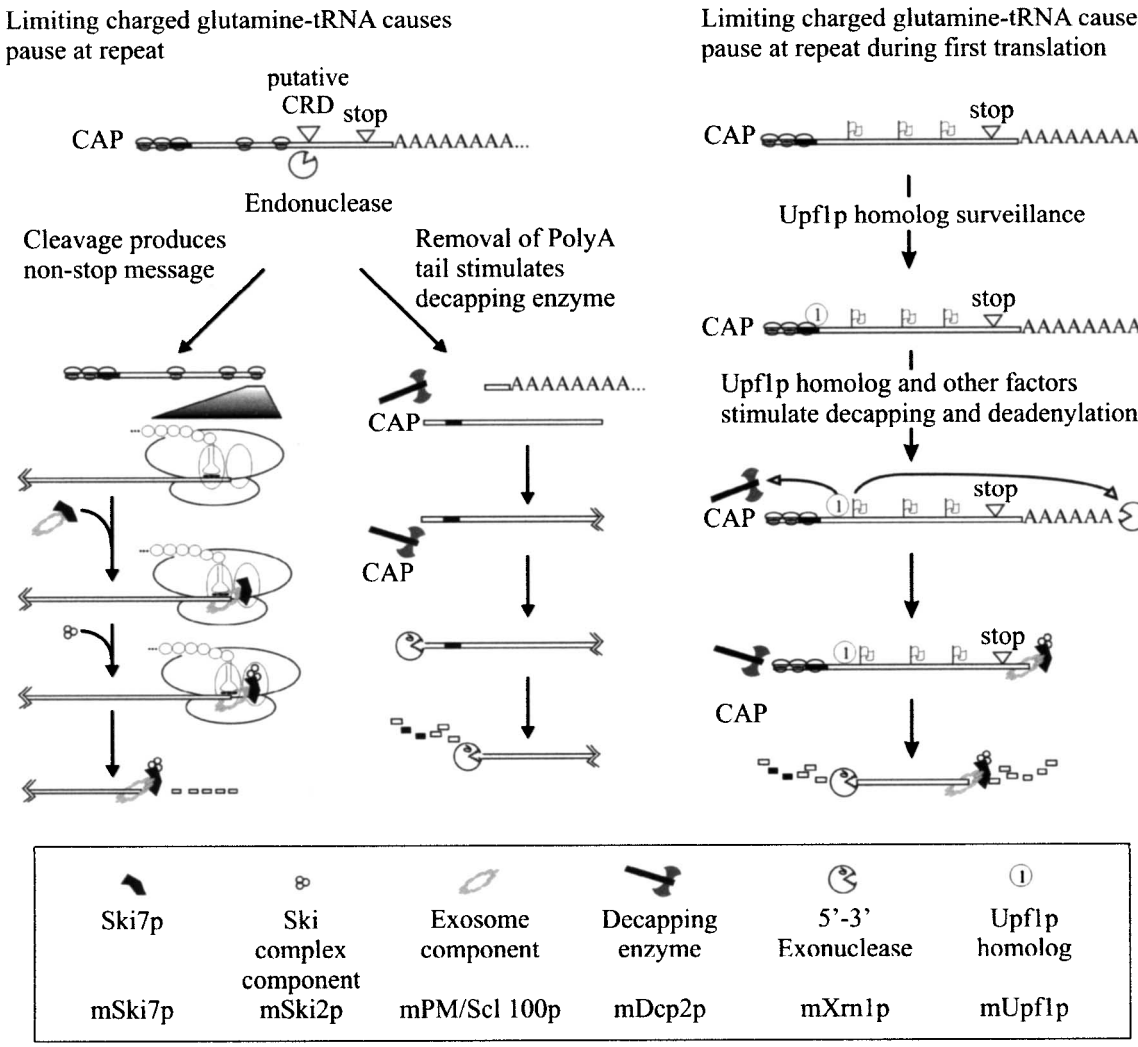

Figure 6. Potential molecular mechanisms of mRNA degradation that might explain the glutamine deprivation effect. Relevant variations of nonstop-mediated decay, decay induced by removal of the poly(A) tail, and nonsense-mediated decay are depicted left to right (24,44). Boxes represent mRNA with filled regions representing long CAG repeats. Homology to the c-*myc* CRD region in *Hdh* mRNA and stop codons are shown as triangles. Double ovals represent ribosomes. Flags in the rightmost pathway depict splicing-dependent markers involved in nonsense-mediated decay. Other symbols and their mouse gene designations are described in box at the bottom.

nisms involving pausing of the ribosome at the repeat might allow endonuclease cleavage to create a nonstop message. Alternately, a pause during the first translation of the mRNA could elicit the nonsense-mediated decay pathway (24,44). These possibilities are outlined in Figure 6. Further work to elucidate the molecular mechanism underlying this effect might allow the development of methods to further stimulate or enhance the specificity of the destruction of long repeat mRNAs.

The complete human genome codes for few mRNAs with a CAG/glutamine repeat longer than 40 and all of these are associated with disease. Thus, a therapeutic window might exist where depletion of expanded CAG/polyglutamine disease mRNAs could be attained without impacting other essential mRNAs. This window may be extremely wide given the possibility that a significant delay in the onset of the polyglutamine diseases might be achieved by mild reductions in disease allele mRNA levels over the decades that typically precede symptoms.

It is possible to reduce blood and tissue glutamine levels by dietary restriction, rigorous exercise, and compounds that inhibit glutamine synthesis (4,42). The glutamine synthesis inhibitor used in this report, methionine sulfoximine, is promising because long-term systemic administration to mice lowered brain glutamine synthetase activity sevenfold without detectable impairment of the cognitive functions that were assayed (4). A more selective mimic of the glutamine deprivation effect might be achieved by one of the known inhibitors of glutaminyl tRNA synthetase (3). The existence of these compounds, the possibility of a wide therapeutic window, and the results presented here argue for the development of therapeutics for polyglutamine disease based on these results. Furthermore, this concept of inducing codon-dependent mRNA destruction by limiting specific charged tRNAs might apply to other diseases whose mechanism depends on high levels of an unwanted disease mRNA.

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